

## Control of *Rhizoctonia solani* with extracts from *Ovidia andina*

### Control de *Rhizoctonia solani* con extractos de *Ovidia andina*

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#### ABSTRACT

New alternatives are needed to control ubiquitous soilborne fungi like *Rhizoctonia solani*. This work evaluates the activity of extracts from *Ovidia andina* on this pathogen. Powdered dried leaves and stems were extracted by maceration with dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), methanol and ethanol. An aqueous extract was obtained by decoction, and the hydrodistillation residual water was the aqueous phase after essences production. A strain of *R. solani* was cultivated on potato dextrose agar supplemented with the extracts, at 1:100 and 1:1000 v/v. The CH<sub>2</sub>Cl<sub>2</sub> extract at 1:100 reduced colony growth (p< 0.0001) and changed hyphal morphology. Five fractions were obtained from the CH<sub>2</sub>Cl<sub>2</sub> extract in a glass column, and TLC analysis showed the presence of coumarins and flavonoids. The CH<sub>2</sub>Cl<sub>2</sub> extract and fractions were diluted in CH<sub>2</sub>Cl<sub>2</sub> or acetone. The efficiency to reduce pathogen growth of fractions 2f and 5f diluted in CH<sub>2</sub>Cl<sub>2</sub> at 1:100 did not differ from the whole extract (p<0.0001). For an *in vivo* test, beetroot seed balls were incubated in soil infested with *R. solani* and treated by immersion in the CH<sub>2</sub>Cl<sub>2</sub> extract at 1:100 for different periods of time. As a result, pathogen colonization was diminished by immersion for 120 s and seed germination was normal (p< 0.0001). Additionally, infested soil was treated with the CH<sub>2</sub>Cl<sub>2</sub> extract, and inoculum concentration estimated using beetroot seed balls as baits diminished (p< 0.0001). These results lead us to conclude that *O. andina* is a source of antifungal components for crop protection against *R. solani*.

#### Keywords

active fractions • bioguided fractionation • CH<sub>2</sub>Cl<sub>2</sub> extract • coumarins and flavonoids characterization • fungal growth

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## RESUMEN

Resulta necesario generar alternativas para el control de hongos patógenos de suelo como *Rhizoctonia solani*. En este trabajo, se evaluó la actividad de extractos de *Ovidia andina* sobre una cepa del patógeno. Se realizaron extracciones a partir de hojas y tallos secos pulverizados, macerados con diclorometano ( $\text{CH}_2\text{Cl}_2$ ), metanol y etanol. Se obtuvo un extracto acuoso por decocción y aguas residuales consistentes en la fase acuosa luego de la producción de esencias. Se cultivó una cepa de *R. solani* en agar papa glucosa suplementado con los extractos diluidos 1:100 o 1:1000 v/v. El extracto  $\text{CH}_2\text{Cl}_2$  1:100 redujo el crecimiento de las colonias ( $p < 0,0001$ ) y alteró la morfología de las hifas. Se obtuvieron 5 fracciones del extracto  $\text{CH}_2\text{Cl}_2$  en una columna de vidrio y mediante TLC se detectó la presencia de cumarinas y flavonoides. Posteriormente, el extracto  $\text{CH}_2\text{Cl}_2$  y sus fracciones se diluyeron en  $\text{CH}_2\text{Cl}_2$  o acetona. La eficiencia de las fracciones 2f y 5f diluidas  $\text{CH}_2\text{Cl}_2$  1:100 para reducir el crecimiento fúngico no difirió de la del extracto completo ( $p < 0,0001$ ). En un experimento *in vivo*, se incubaron glomérulos de remolacha en suelo infestado con *R. solani* y se sumergieron en el extracto  $\text{CH}_2\text{Cl}_2$  1:100 durante tiempos variables. La colonización fúngica disminuyó mediante la inmersión durante 120 segundos, sin afectar la germinación ( $p < 0,0001$ ). Adicionalmente, se trató suelo infestado con el extracto  $\text{CH}_2\text{Cl}_2$  y disminuyó la concentración de inóculo del patógeno estimada utilizando glomérulos de remolacha como trampas ( $p < 0,0001$ ). Estos resultados permiten concluir que *O. andina* es una fuente de componentes antifúngicos para la protección vegetal contra *R. solani*.

### Palabras clave

fracciones activas • fraccionamiento bioguiado • extracto  $\text{CH}_2\text{Cl}_2$  • caracterización de cumarinas y flavonoides • crecimiento fúngico

## INTRODUCTION

*Rhizoctonia solani* J. G. Kühn is a causative agent of seedling damping-off and crown and root rot diseases, with a total of 2,593 fungus-host combinations recorded around the world (10). This species has been identified on a wide range of hosts that includes vegetables, oil crops, forest trees, forages, ornamentals, fruits, and cereals (21). Pathogenic strains of *R. solani* may be transmitted by soil and also by seeds of many hosts (27). Although the most important control measures are cultural, chemicals are still important tools to reduce the damages caused by *R. solani* (32). However, the concerns about the

safety and environmental impact of chemicals have focused research on alternative strategies to manage plant diseases (26).

Plants constitute a potential source of chemical structures, especially secondary metabolites (1, 13) that could be appropriate for crop protection, in an integrated management framework (3). The shrub *Ovidia andina* (Poepp. and Endl.) Meisn. belongs to the Thymelaeaceae and is commonly known as pillo-pillo (36) or traro-voqui (30). This species occurs from 700 to 2,300 m elevation in central Chile and along the adjacent Andean slopes of Argentina (19).

The Mapuche community uses it as a medicinal plant (17). As many other native species, *O. andina* may be of interest to be evaluated for plant protection. The objectives of this work were to obtain extracts and purified fractions from *O. andina*, to evaluate their efficiency to control *R. solani* and to characterize the bioactive substances.

## MATERIALS AND METHODS

### Plants and pathogen

*O. andina* plants were collected at Cerro Centinela (43°29'0" S 71°35'53" W), located in Esquel, province of Chubut, Argentina in May. An entry coded as BAF 17501 was deposited at the Herbarium of the Museo de Farmacobotánica J. A. Domínguez, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. The plant material was collected by Dr. Silvia González (Universidad Nacional de la Patagonia San Juan Bosco, Esquel), and the identification was confirmed by Dr. Nidia Hansen (INTA, Esquel).

The pathogenic isolate O-IM-1, belonging to *Rhizoctonia solani* AG-4 HG-II and isolated in Buenos Aires (25) is part of a fungal collection of the Cátedra de Fitopatología, Facultad de Agronomía, Universidad de Buenos Aires. It was maintained on potato dextrose agar (PDA, Merck) and used throughout all this study. Beetroot (*Beta vulgaris* L., Amaranthaceae) was chosen as bait to estimate inoculum density of *R. solani*, as well as target for disease control assays. Beetroot fruits are the organs used to initiate the crop and are commonly referred to as seeds or seed balls.

### Extracts and extract characterization

Plants of *O. andina* were dried by forced air at room temperature, and leaves and stems were ground with a blade mill.

The powder (100 g) was soaked in dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) over a 24 h period, and filtered through Whatman No. 1 filter paper (gravity filtration). The protocol was repeated three times, and then the CH<sub>2</sub>Cl<sub>2</sub> extracts were pooled and taken to dryness in a rotary evaporator. The plant drug previously extracted with CH<sub>2</sub>Cl<sub>2</sub> was air dried over a 24 h period, soaked in methanol for 24 h, and finally filtered using Whatman No. 1 filter paper (gravity filtration). This was repeated three times. Subsequently, the methanolic (MeOH) extracts were pooled, dried in a rotary evaporator and lyophilized.

A 70% v/v ethanolic (EtOH) extract was obtained as follows: 12 g of powdered and dried aerial organs were placed in an Erlenmeyer flask, extracted with 400 mL of 70% v/v ethanol under agitation with a magnetic stir bar for 30 min, and filtered through Whatman No. 1 filter paper. This procedure was repeated three times. The extracts were pooled, dried in a rotary evaporator and lyophilized.

An aqueous (Aq) extract was obtained by boiling 20 g of powdered and dried leaves and stems in an Erlenmeyer flask containing 400 mL of distilled water for 20 min. The decoction was allowed to cool to 40-45°C, filtered through a Whatman No. 1 filter paper (gravity filtration) and the volume restored to 400 mL by adding distilled water (9), and lyophilized (FTS Flexi-Dry lyophilizer).

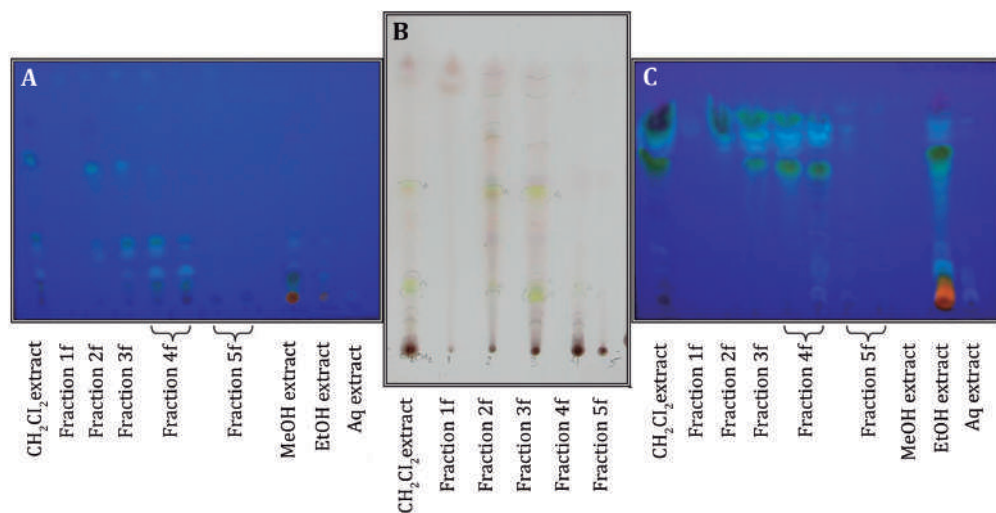
Additionally, the aqueous phase that remained after hydrodistillation to obtain essences from *O. andina* (9) was filtered, reduced in volume in a rotary evaporator, lyophilized and named hydrodistillation residual water (Hrw).

After analysis by means of the first bioassay (test 1) described later, two other assays (tests 2 and 3) were employed to quantify the activities of the CH<sub>2</sub>Cl<sub>2</sub> extract fractions.

The extract (90 mg) was fractionated at atmospheric pressure in an open glass column (4 cm x 3.5 cm), employing Macherey-Nagel silica gel 60 (0.063-0.2 mm / 70-230 mesh ASTM for column chromatography) as stationary phase, and solvents or solvent mixtures of increasing polarity as mobile phases (toluene, toluene + ethyl ether (7:3), toluene + ethyl ether (1:1), ethyl ether, ethyl ether + methanol (1:1), methanol).

Fractioning was repeated four times, and the fractions eluted through the columns were analyzed by thin layer chromatography (TLC). Similar fractions from the four columns were collected, obtaining seven fractions (1 to 7). All the extracts and fractions were analyzed

through the following TLC systems: I) Stationary phase (SP): silica gel, Mobile phase (MP): toluene:ethylacetate (8:2); II) SP: silica gel; MP: CH<sub>2</sub>Cl<sub>2</sub>:MeOH (9:1). The chromatograms were developed with Natural Product reagent (NP) Fluka, a polyphenol reagent, and observed under natural and UV 366 nm light (TLC systems I and II) (figure 1A, figure 1C). The fractions 4 and 5 as well as the fractions 6 and 7, with similar profiles in both systems were pooled in the final fractions 4f and 5f respectively. This fractionation was guided according to antifungal activity. The final fractions were named 1f to 5f. The TLC system I was also developed with 50% v/v H<sub>2</sub>SO<sub>4</sub>, a universal reagent, and observed under natural light (figure 1B) (33).



CH<sub>2</sub>Cl<sub>2</sub>: dichloromethane extract, MeOH: methanolic extract, EtOH: ethanolic extract, Aq: aqueous extract, Fractions 1f to 5f: final fractions of the CH<sub>2</sub>Cl<sub>2</sub> extract.

CH<sub>2</sub>Cl<sub>2</sub>: extracto diclorometánico, MeOH: extracto metanólico, EtOH: extracto etanólico, Aq: extracto acuoso, Fracciones 1f a 5f: fracciones finales del extracto CH<sub>2</sub>Cl<sub>2</sub>.

**Figure 1.** Thin layer chromatography (TLC) of the extracts and fractions obtained from *Ovidia andina*. A. TLC system I, developed with natural products (NP) reagent. B. TLC system I, developed with sulfuric acid - ethanol 50% v/v reagent. C. TLC system II, developed with NP reagent.

**Figura 1.** Cromatografía en capa fina (TLC) de los extractos y fracciones obtenidos de *Ovidia andina*. A. sistema TLC I revelado con reactivo NP. B. sistema TLC I revelado con ácido sulfúrico - etanol 50% v/v. C. sistema TLC II revelado con reactivo NP.

### **Pathogen control**

#### *Colony growth*

Three assays (tests 1 to 3) were performed. In test 1, *R. solani* strain O-IM-1 was grown on PDA supplemented with different extracts obtained from *O. andina*. For that purpose, the CH<sub>2</sub>Cl<sub>2</sub> extract was diluted in CH<sub>2</sub>Cl<sub>2</sub>; the MeOH, EtOH and Aq extracts were diluted in 80% v/v ethanol, and the HrW was diluted in water, all at 1:10 and 1:100 v/v (controls: CH<sub>2</sub>Cl<sub>2</sub>, ethanol, water). One mL of each dilution was pipetted into a Petri dish, and all the plates were kept open under laminar flow, time enough to allow the evaporation of the solvent and consequently avoid its interference (8). Ten mL of molten PDA were then poured into each plate, so that final extract dilutions were 1:100 and 1:1000. Plugs of 15 mm diameter were cut from the edge of colonies of *R. solani* of 5 days on PDA and used as a source of inoculum, and one plug was placed in the center of each plate after the PDA had solidified. The plates were randomly distributed in an incubator, at 22°C. Three replicates were done per treatment and the experiment was conducted three times. Pathogen radial growth was registered daily and the inhibition was calculated (31). Pathogen hyphae were observed with a compound microscope.

For test 2, the pathogen was grown on PDA supplemented with the CH<sub>2</sub>Cl<sub>2</sub> extract and fractions 1f to 5f diluted in CH<sub>2</sub>Cl<sub>2</sub> at 1:100 (control: CH<sub>2</sub>Cl<sub>2</sub>). For test 3, the pathogen was grown on PDA supplemented with the CH<sub>2</sub>Cl<sub>2</sub> extract and fractions 1f to 5f diluted in acetone at 1:100 (control: acetone). The protocol for tests 2 and 3 was identical to the one detailed for test 1.

#### *Seed ball colonization and seed germination*

A sample of field soil was placed in 4.4 dm<sup>3</sup> metal containers, autoclaved at 1 atm for 1 h and allowed to cool. Plugs

of 1 cm<sup>3</sup> from the edges of a colony of *R. solani* on PDA were placed on the surface of the soil (0.1% v/v), the containers were sealed with aluminum foil and incubated at room temperature over a period of 5 days (25) so as to obtain inoculum for the experiments described below. Groups of fifty beetroot seed balls were evenly distributed over 32 g of the infested soil in a Petri dish, and covered with an additional 32 g of infested soil. After 48-h incubation at 26°C, the seed balls were recovered, washed for 5 min under running tap water in a colander, and excess water was blotted with paper towels. Samples of 10 fungal-exposed seed balls were immersed into 2 mL of the CH<sub>2</sub>Cl<sub>2</sub> extract 1:100 diluted in CH<sub>2</sub>Cl<sub>2</sub> (controls CH<sub>2</sub>Cl<sub>2</sub>, water) contained in test tubes and shaken with a vortex shaker. Three replications were done per treatment and the assay was repeated twice. Immersion times were 30, 60, 90, 120 and 150 s. The seed balls were plated on PDA (pH4) (16) placing 50 seed balls in each plate. The number of seed balls with emerging hyphae of *R. solani* was determined by observations under a stereomicroscope after 24 h. Each seed ball with emerging *R. solani* hyphae was counted as positive. Colonies of *R. solani* that developed from beetroot seed balls were photographed 24 h after evaluation under the stereo microscope. A previous assay was done to verify that *R. solani* was not present in the seed balls before the treatments, by a modification of Ko and Hora (16) method: sterilized soil was used instead of colonized soil, and the seed balls were immersed in water for 30, 60, 92, 120 and 150 s. As described above, the seed balls were plated on PDA and the number of seed balls with emerging hyphae of *R. solani* was determined by observations under the stereomicroscope after 24 h.

Another assay was done to verify absence of dormancy in the seeds (results not shown), so as to determine the need of a 2 h washing treatment to eliminate water soluble germination inhibitors located in the pericarp. A germination test was followed in order to evaluate the effect of the CH<sub>2</sub>Cl<sub>2</sub> extract on beetroot. Seed balls were immersed for 120 s in the CH<sub>2</sub>Cl<sub>2</sub> extract 1:100 (controls: CH<sub>2</sub>Cl<sub>2</sub>, water), placed on moistened, pleated sheets of tissue paper and incubated at 20°C (7). Five replicates were done per treatment and the experiment was conducted three times. Percentage of germination was evaluated at 14 and 21 days.

#### Soil infestation

A sample of field soil was infested with the strain of *R. solani* and placed in Petri dishes, following the method already described. Beetroot seed balls were placed in the middle of the two layers of 32 g of soil, as baits for a further estimation of the inoculum concentration of *R. solani* in the soil treated with the CH<sub>2</sub>Cl<sub>2</sub> extract 1:100. One mL of the CH<sub>2</sub>Cl<sub>2</sub> extract was pipetted into each Petri dish just before placing the soil (controls: CH<sub>2</sub>Cl<sub>2</sub>, water). Five replications were done per treatment and the assay was repeated twice. After 48 h of incubation at 26°C, the seed balls were recovered, washed and incubated on PDA (10 seed balls plate<sup>-1</sup>) before observation

under the stereomicroscope. Each seed ball with emerging *R. solani* hyphae was counted as positive. Inoculum concentration in the soil was estimated as the mean number of fungal propagules gram<sup>-1</sup> of soil (16).

#### Statistical analysis

All the tests were designed at random. Infostat software (FCA-UNC, Córdoba) was used for statistical calculations. Split-plot methodology was chosen for two-factor ANOVAs when time was one of the factors; and Satterwaite procedure was used for multiple comparisons when interactions between factors were significant (seed germination, pathogen growth). DGC test was chosen for one-factor analyses (pathogen growth), while Kruskal Wallis non parametric test was used for the analysis of seed ball colonization.

## RESULTS

#### Extracts and extract characterization

Table 1 summarizes the quantities of extracts obtained from leaves and stems of *O. andina*, and their respective yields (g extract 100g<sup>-1</sup> leaves and stems). The TLC analysis of the CH<sub>2</sub>Cl<sub>2</sub>, MeOH, EtOH and Aq extracts and the fractions 2f to 5f of the CH<sub>2</sub>Cl<sub>2</sub> extract showed the presence of coumarins and flavonoids (figure 1, page 358).

**Table 1.** Extracts obtained from the samples of *Ovidia andina* with different solvents.

**Tabla 1.** Extractos obtenidos de las muestras de *Ovidia andina* con solventes diferentes.

Extract	Leaves + stems (g)	Extract (g)	Yield <sup>1</sup> (%)
Cl <sub>2</sub> CH <sub>2</sub> (dichloromethanic)	100.0	4.0	4.0
MEOH (methanolic) <sup>2</sup>	100.0	7.4	7.4
EOH (ethanolic)	12.2	2.1	17.2
Aq (aqueous)	20.1	2.9	14.4

<sup>1</sup>g extract 100g<sup>-1</sup> leaves and stems. <sup>2</sup>after extraction with CH<sub>2</sub>Cl<sub>2</sub>.

<sup>1</sup>g extracto 100g<sup>-1</sup> hojas y tallos. <sup>2</sup>luego de la extracción con CH<sub>2</sub>Cl<sub>2</sub>.



The coumarins were observed as light blue spots and the flavonoids as yellow spots when treated with NP reagent and viewed under UV light at 366 nm, and the flavonoids were observed as yellow spots when revealed with 50% H<sub>2</sub>SO<sub>4</sub> reagent.

### Pathogen control

#### Colony growth

Interactions between treatments and times of evaluation were significant ( $p < 0.0001$ ) in the three tests.

Figure 2 shows the results of multiple comparison tests for the growth of *R. solani* at the end of test 1.

The CH<sub>2</sub>Cl<sub>2</sub> extract at 1:100 inhibited fungal growth, and the rest of the treatments did not differ from the control.

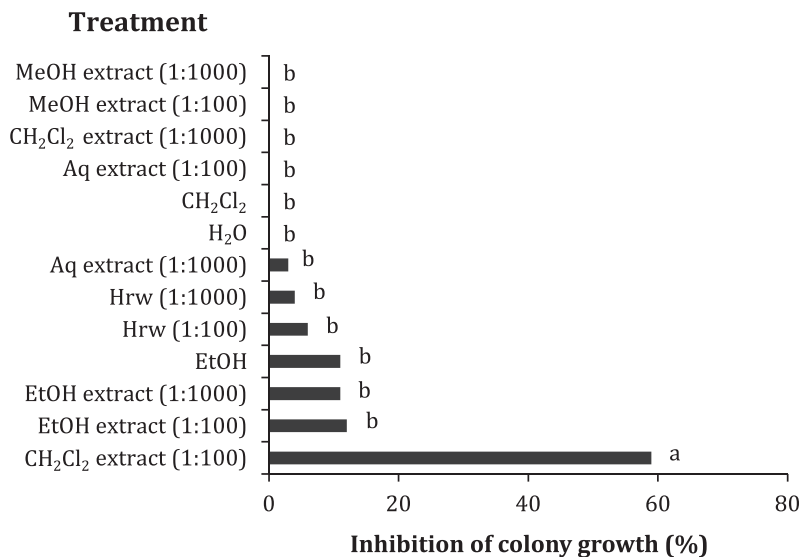
Hyphae appeared widened, swollen and contained discrete granules following treatments with the CH<sub>2</sub>Cl<sub>2</sub> extract at 1:100 (figure 3, page 362).

In test 2, the best efficiency was observed for the CH<sub>2</sub>Cl<sub>2</sub> extract, fraction 2f and fraction 5f; followed by fractions 3f and 1f; all diluted in CH<sub>2</sub>Cl<sub>2</sub> (figure 4, page 362).

In test 3, the best efficiency was observed for the CH<sub>2</sub>Cl<sub>2</sub> extract, followed by fraction 2f and fraction 3f; all diluted in acetone (figure 5, page 363).

#### Seed ball colonization and seed germination

The sample of beetroot seed balls tested for the presence of the pathogen showed no evidence of *R. solani* previous to the seed ball colonization test.

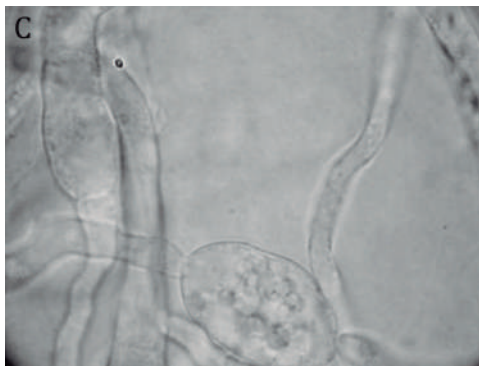


Different letters indicate differences between treatments. DGC test,  $\alpha$ : 0.05.

Letras diferentes indican diferencias entre tratamientos. Prueba DGC,  $\alpha$ : 0,05.

**Figure 2.** Growth of *R. solani* on potato dextrose agar supplemented with the extracts obtained from *Ovidia andina*, diluted in CH<sub>2</sub>Cl<sub>2</sub>.

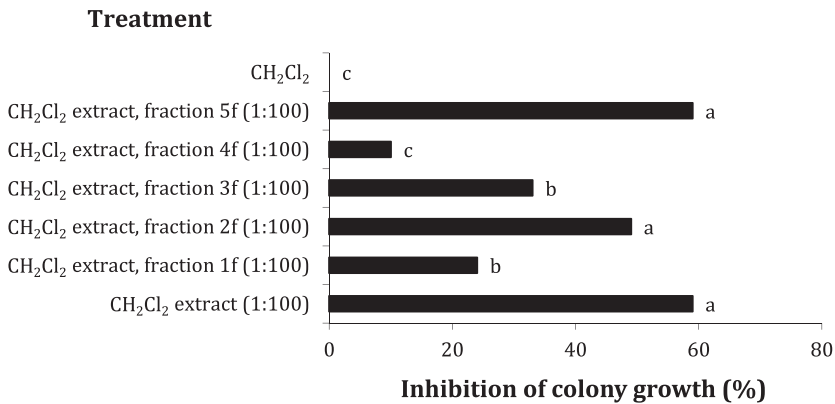
**Figura 2.** Crecimiento de *R. solani* en agar papa glucosa suplementado con los extractos de *Ovidia andina* diluidos en CH<sub>2</sub>Cl<sub>2</sub>.



A. Control. B. Widened hypha and granules. C. Swollen hyphae.  
 A. Testigo. B. Hifa ensanchada y presencia de gránulos. C. Hifa hinchada.

**Figure 3.** Microscope observations of hyphae of *R. solani* after treatments with the dichloromethanic ( $\text{CH}_2\text{Cl}_2$ ) extract of *Ovidia andina*.

**Figura 3.** Observaciones al microscopio de hifas de *R. solani* luego del tratamiento con el extracto diclorometánico ( $\text{CH}_2\text{Cl}_2$ ) de *Ovidia andina*.



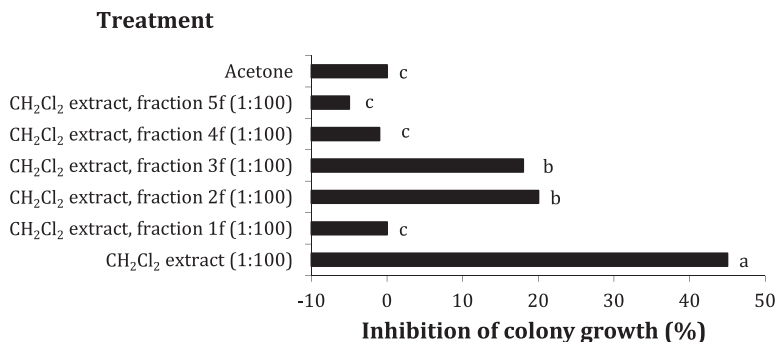
Different letters indicate differences between treatments. DGC test,  $\alpha$ : 0.05.

Letras diferentes indican diferencias significativas entre tratamientos. Prueba DGC,  $\alpha$ : 0,05.

**Figure 4.** Growth of *Rhizoctonia solani* on PDA supplemented with the  $\text{CH}_2\text{Cl}_2$  extract from *Ovidia andina* and its fractions, diluted in  $\text{CH}_2\text{Cl}_2$ .

**Figura 4.** Crecimiento de *Rhizoctonia solani* en agar papa glucosa suplementado con el extracto  $\text{CH}_2\text{Cl}_2$  de *Ovidia andina* y sus fracciones, diluidas en  $\text{CH}_2\text{Cl}_2$ .





Different letters indicate differences between treatments. DGC test,  $\alpha$ : 0.05.

Letras diferentes indican diferencias significativas entre tratamientos. Prueba DGC,  $\alpha$ : 0,05.

**Figure 5.** Growth of *Rhizoctonia solani* on potato dextrose agar supplemented with the CH<sub>2</sub>Cl<sub>2</sub> extract from *Ovidia andina* and its fractions, diluted in acetone.

**Figura 5.** Crecimiento de *Rhizoctonia solani* en agar papa glucosa suplementado con el extracto CH<sub>2</sub>Cl<sub>2</sub> de *Ovidia andina* y sus fracciones diluidos en acetona.

Levels of colonization by *R. solani* were significantly diminished following immersion in the CH<sub>2</sub>Cl<sub>2</sub> extract diluted in CH<sub>2</sub>Cl<sub>2</sub> for 120 and 150 s (figure 6, page 364).

Beetroot germination was delayed by immersion in the CH<sub>2</sub>Cl<sub>2</sub> extract, as it was reduced at day 14, but similar to the control at day 21 (table 2, page 364). Seedlings developed from seed balls treated with CH<sub>2</sub>Cl<sub>2</sub> were not visibly different from the control.

#### Soil infestation

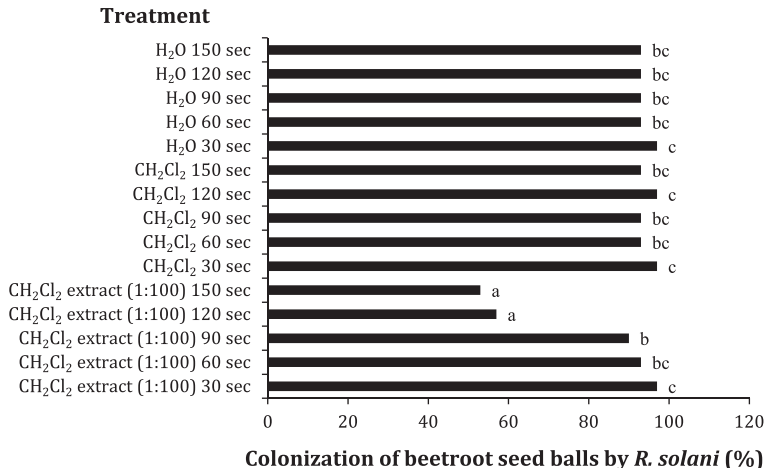
Inoculum density of *R. solani* estimated as the number of propagules in the soil diminished in the extract treatments (table 2, page 364). Figure 7 (page 365) shows the colonies of *R. solani* that developed on PDA from beetroot seeds 24 h after evaluation under the stereomicroscope (48 h incubation).

## DISCUSSION

The samples of leaves and stems of *O. andina* yielded the dichloromethane, methanolic, ethanolic and aqueous extracts as well

as the hydrodistillation residual water used in the experiments. Among all the extracts evaluated, the CH<sub>2</sub>Cl<sub>2</sub> extract at 1:100 was the only one able to diminish the growth of *R. solani*, inhibiting colony area at 59%. With CH<sub>2</sub>Cl<sub>2</sub> as diluent, the CH<sub>2</sub>Cl<sub>2</sub> extract and fractions 2f and 5f showed antifungal activity; while when acetone was used as the solvent, the CH<sub>2</sub>Cl<sub>2</sub> extract showed the highest activity, followed by fractions 2f and 3f. Even if fraction 2f was active in both diluents, it was more suppressive when diluted in CH<sub>2</sub>Cl<sub>2</sub>. It is known that the effectiveness of plant extracts depends on the presence of active components, which can be obtained with different solvents and extraction techniques. The activity of fraction 5f was markedly higher when diluted in CH<sub>2</sub>Cl<sub>2</sub>, as it had been the extraction solvent, than when diluted in acetone.

This behavior would indicate that a complete dilution of the CH<sub>2</sub>Cl<sub>2</sub> extract is hindered by acetone, a solvent with higher polarity than CH<sub>2</sub>Cl<sub>2</sub>. The differences between assays 2 and 3 in the activity observed for the fractions may be related to their better dilution in CH<sub>2</sub>Cl<sub>2</sub>.



Different letters indicate differences between treatments. Kruskal-Wallis test,  $\alpha$ : 0.05.  
 Letras diferentes indican diferencias entre tratamientos. Prueba Kruskal-Wallis,  $\alpha$ : 0,05.

**Figure 6.** Beetroot seeds infected by *R. solani* in dichloromethanic (CH<sub>2</sub>Cl<sub>2</sub>) extract-treatment.

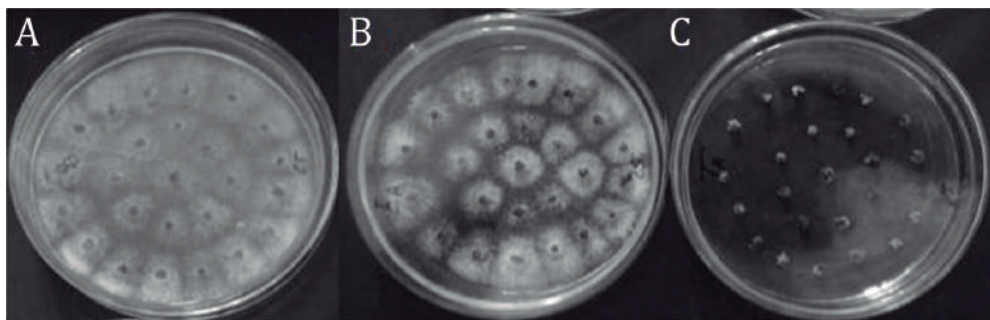
**Figura 6.** Semillas de remolacha infectadas con *R. solani* en tratamientos con extracto diclorometánico (CH<sub>2</sub>Cl<sub>2</sub>).

**Table 2.** Beetroot germination and soil infestation after seed ball and soil treatments with the dichloromethanic (CH<sub>2</sub>Cl<sub>2</sub>) extract from *Ovidia andina*.

**Tabla 2.** Germinación y grado de infestación de suelo luego de aplicar extracto diclorometánico (CH<sub>2</sub>Cl<sub>2</sub>) de *Ovidia andina* a glomérulos de remolacha y suelo.

Treatment	Germination of beetroot seeds <sup>1</sup> (%)		Population of <i>Rhizoctonia solani</i> in soil <sup>2</sup> (No propagules g <sup>-1</sup> )
	day 14	day 21	
H <sub>2</sub> O	87a	87a	0.020a
CH <sub>2</sub> Cl <sub>2</sub>	86a	86a	0.010a
CH <sub>2</sub> Cl <sub>2</sub> extract 1:100	51b	84a	0.002b

Different letters within a column indicate differences between treatments (<sup>1</sup>Satterwaite test, <sup>2</sup>DGC test,  $\alpha$ : 0.05).  
 Letras diferentes dentro de cada columna indican diferencias entre tratamientos (<sup>1</sup>prueba Satterwaite, <sup>2</sup>prueba DGC,  $\alpha$ : 0,05).



**Figure 7.** Colonies of *Rhizoctonia solani* on potato dextrose agar, developed from beetroot seed balls incubated in soil treated with A. water. B. dichloromethane ( $\text{CH}_2\text{Cl}_2$ ). C.  $\text{CH}_2\text{Cl}_2$  extract.

**Figura 7.** Colonias de *Rhizoctonia solani* en agar papa glucosa, desarrolladas a partir de glomérulos de remolacha incubados en suelo tratado con A. agua. B. diclorometano ( $\text{CH}_2\text{Cl}_2$ ). C. extracto  $\text{CH}_2\text{Cl}_2$ .

According to the results, fractions 2f and 5f would contain the most active compounds present in the  $\text{CH}_2\text{Cl}_2$  extract. The higher or similar activity of the extract compared with its fractions could be attributed to compounds' synergy, which may potentiate each other's action. A similar effect had been observed (4) for a combination of a coumarin with other compounds from fruits of *Melia azedarach*, that strengthened individual antifungal effects against *Fusarium verticillioides*.

Phenolics are a large class of secondary metabolites produced by plants, that include defense-related flavonoids and coumarins. TLC is useful for the analysis of phenolics, and silica gel is the most frequently used adsorbent (33). The chromatographic analysis of the fractions obtained from the  $\text{CH}_2\text{Cl}_2$  extract showed the presence of flavonoids and coumarins, which could be related to the antifungal behavior observed, in coincidence with Montagner *et al.* (2008). Free flavonoid aglicones exuded by plant tissues may be washed from the surface with nonpolar

solvents, while more polar glycosidic conjugates dissolve in polar solvents (28). This differential behavior may indicate the type of compound that is active in the studied  $\text{CH}_2\text{Cl}_2$  extract from *O. andina*.

Coumarins also constitute an important group of natural compounds (18) derived from the lactone of the o-hidroxi cinnamic acid, and may be free or glycosilated in the plants (5). The successive use of solvents with increasing polarity has been effective for their extraction and isolation (35). In this work the plant material was macerated with  $\text{CH}_2\text{Cl}_2$  in order to obtain the  $\text{CH}_2\text{Cl}_2$  extract, which would be expected to contain low polar and moderately polar compounds present in this species. The vegetable drug was then dried and extracted with methanol. Consequently, the MeOH extract contains the polar compounds present. In addition to a  $\text{CH}_2\text{Cl}_2$  extract, a hydroalcoholic extract and a decoction were evaluated, given that the solvents used to obtain them are the most frequently used because of their low toxicity on human beings (6).

The potential of wild plants as sources of phenolics to be utilized as natural anti-infective agents in the agricultural industry remains largely untapped (24). They are barriers to fungal penetration in plant tissues (11), and have provided control of *R. solani* (2, 12, 18, 34). The current work reports morphological alterations and reduced colony growth of *R. solani* in the presence of a dichloromethane extract of *O. andina* for the first time. Coincidentally, Plodpai *et al.* (2013) observed globular structures in cells of *R. solani* caused by a dichloromethane extract from *Desmos chinensis*. The flavonoids and coumarins we have detected in the extract might be some of the active antifungal compounds. The activity of the fractions obtained from the CH<sub>2</sub>Cl<sub>2</sub> extract differed from each other. This behavior may be attributed to the purity of each fraction or to the synergism among its compounds (14).

Seeds of vegetables, ornamentals or field crops contaminated or infected with *R. solani* may decay or may produce infected seedlings from which the fungus spreads into the soil or to adjacent seedlings. It was proved in this work that *R. solani* contamination of beetroot seed balls may be reduced by immersion in the CH<sub>2</sub>Cl<sub>2</sub> extract diluted in CH<sub>2</sub>Cl<sub>2</sub> for 120 seconds. The extract delayed germination when evaluated at day 14, but was not significantly different from the control by 21 days after immersion in CH<sub>2</sub>Cl<sub>2</sub> extract. The germination of *B. vulgaris* is very sensitive to chemical conditions in the seed bed (15). Talukder *et al.* (29) observed a delay in germination after exposing seeds of *B. vulgaris* to plant extracts. This behavior could be attributed to allelopathy in the extract compounds (20). In this work, the phenomenon was temporary.

Like many other pathogens, *R. solani* has the ability to grow on or through the soil. It was demonstrated that the CH<sub>2</sub>Cl<sub>2</sub> extract from *O. andina* inhibits fungal growth on agar media and reduces the inoculum in the soil. *R. solani* is not transmitted by beetroot seeds but is likely to be transported by seed balls after contact with infested soil or plants. The baiting method used throughout this work proved to be sensitive for detecting and quantifying *R. solani* in extract treatments on seed balls and soil. The use of beetroot seed balls as well as wooden toothpicks (22) as baits, are simple and inexpensive techniques that can be used for the detection of *Rhizoctonia* spp. in different studies.

These results constitute the basis to continue the phytochemical research, with the aims of obtaining pure active compounds and fulfilling studies on mechanisms of action on the pathogen. Additionally, further studies could be done to test the effect of the extract on other isolates of the pathogen, which may include other anastomosis groups as well as different soilborne species.

## CONCLUSIONS

The native species *O. andina* has arisen as a promising source of anti-fungal compounds. This work demonstrates that the dichloromethane extract of *Ovidia andina* reduces colonization of the beetroot fruits after exposing to high levels of *Rhizoctonia solani* in the soil and controls soil infestation; and that is harmless to the seeds and seedlings.

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